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Article

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ABSTRACT:

Vitamin K food composition data have historically been limited to plant-based phylloquinone (vitamin K_1). The purpose of this study was to expand analysis of vitamin K to animal products, and measure phylloquinone and 10 forms of menaquinones (vitamin K_2) in processed and fresh-cut pork products. Nationally representative samples of processed pork products (n=28) were obtained through USDA's National Food and Nutrition Analysis Program, and fresh pork (6 cuts; n=5 per cut) and bacon (n=4) were purchased from local retail outlets. All samples were analyzed by HPLC (phylloquinone and menaquinone-4) and APCI-LC-MS (menaquinone-5 to menaquinone-13). Although low in phylloquinone (<2.1±0.5 µg phylloquinone/100 g), all processed pork products and fresh pork cuts contained menaquinone-4, menaquinone-10 and menaquinone-11 (range: 35.1±11.0-534±89.0 µg menaquinones/100 g). The total menaquinone contents of processed pork products were correlated with fat contents (r=0.935). In summary, processed and fresh-cut pork products are a rich dietary source of menaquinones that are currently unaccounted for in assessment of vitamin K in the food supply.

Keywords:

Vitamin K; phylloquinone; menaquinones; pork; sausage; food composition

INTRODUCTION

Vitamin K is necessary for synthesis of vitamin K-dependent proteins which have multiple physiological functions, including regulation of blood clotting.[1-3] Dietary sources of vitamin K are found in two natural forms, phylloquinone and menaquinones, both of which share the common structure, 2-methyl-1,4-napthoquinone. Phylloquinone, the most abundant form of vitamin K in the diet, is present mainly in dark green vegetables.[2] The majority of menaquinones are synthesized by bacteria, and are thought to be abundant in certain fermented foods.[4, 5] Whereas phylloquinone is widely distributed in food supply, little is known about the various menaquinone forms in foods.[6, 7] A novel method employing high-performance liquid chromatography–mass spectrometry with atmospheric pressure chemical ionization (LC–APCI–MS) now allows for simultaneous quantification of 11 vitamin K vitamers that can be applied in various food matrices.[8]

The menaquinones differ in structure from phylloquinone in their 3-substituted lipophilic side chain, and are designated by the number of isoprenoid units, i.e. -n. Menaquinone-4 is a unique form of vitamin K as it cannot be synthesized by bacteria. Instead, it is converted from phylloquinone or menadione endogenously in mammals.[9, 10] Menadione, a provitamin form of vitamin K, is commonly used in animal diets as the sole dietary source of vitamin K. There is also speculation that longer chain menaquinones, such as menaquinone-7, can also be converted to menaquinone-4.[11]

Recent reports from Europe attribute unique heart health benefits to menaquinone forms obtained from the diet.[12] Inverse associations have been reported between menaquinone intake and severe aortic calcification,[12, 13] the risk of coronary heart disease (CHD),[3] and the risk of CHD mortality and all-cause mortality [13] in community-based cohorts. In the Dutch PROSPECT study cohort, the reduction in CHD risk was mainly driven by menaquinone-7, menaquinone-8 and menaquinone-9 consumption.[3] Because menaquinone forms have not been systematically analyzed in U.S. foods nor have menaquinone intakes been estimated in the U.S. population, these observations have yet to be substantiated in the U.S. The purpose of this study was to quantify the amounts of menaquinones using state-of-the-art LC-MS technology, in representative samples of fresh and processed pork products commonly consumed in the

U.S. diet.

MATERIAL AND METHODS

Samples

The processed pork samples used in this study were provided by United States Department of Agriculture (USDA) Nutrient Data Laboratory (NDL), which conducts the National Food and Nutrition Analysis Program (NFNAP).[14-16] The food samples were first delivered to the Food Analysis Laboratory Control Center at Virginia Tech in Blacksburg, Virginia, for preparation of aliquots and quality-control materials. The processed pork and quality-control samples were then delivered on dry ice to the Vitamin K Laboratory at Tufts University and stored at -80^{o}C until analysis. The NFNAP infrastructure incorporates a nationally-representative sampling approach,[15] approved analytical methods, and a rigorous quality assurance scheme.

Fresh-cut pork products were purchased from 4 supermarkets in Eastern Massachusetts. Six parts of fresh-cut pork, including chops, chops with bone, back ribs with bone, shoulder blade with bone, tenderloin and St. Louis-style cut ribs, were selected (**Figure 1**). Each part included 5 samples that were purchased from either different supermarkets or different days in the same supermarket. All purchased fresh-cut pork samples were transported to the Vitamin K Laboratory at Tufts University, Boston, within one hour, and stored at -80°C until analysis. All samples were analyzed within one month after sample collection. The sample collection, preparation and analysis are summarized in **Figure 2**.

The phylloquinone and menaquinone-4 to menaquinone-13 contents of all samples were initially determined by APCI-LC-MS, as described previously.[8] Deuterium-labeled phylloquinone was used as internal standard. The phylloquinone and menaquinone-4 concentrations of the pork products were below the low limit of detection (LOD) of the APCI-LC-MS assay (phylloquinone: 2.2 μg/100 g ; menaquinone-4: 2.1 μg/100 g), so the phylloquinone and menaquinone-4 contents of samples were subsequently re-analyzed by HPLC using procedures described elsewhere.[17] The LOD of phylloquinone and menaquinone-4 for this HPLC assay was 0.2 μg/100 g. The $K_{1,25}$ was added as internal standard. In preparing fresh-cut pork samples for vitamin K analysis, any bone was first removed, and then each sample was chopped and grounded using a Waring Pro meat grinder (East Windsor, NJ). About 50 g of ground sample was homogenized with 200 mL PBS using a 3HPP commercial blender (Cleanblend, San Diego, CA). Vitamin K extraction procedures were the same for both methods. Briefly, homogenized processed and fresh-cut pork products were weighed, and 0.15 g samples were used for analysis. Water and hexane/isopropanol (3:2 v/v) were added and the samples were sonicated for 1 min. Then the samples were vortexed for 3 min and centrifuged. The top solvent was removed and the samples were dried under N_2. The re-concentrated samples were further purified through solid-phrase extraction using silica columns. The eluted samples were dried again and ready for analysis by APCI-LC-MS for menaquinone 5-13 and HPLC for phylloquinone and menaquinone-4.

All samples were analyzed in duplicate. The assay was repeated if the CV of duplicates was over 15%. In the absence of standards for vitamin K forms in foods, an in-house control sample (consisting of an aliquot of baby food chicken vegetable dinner, Beechnut, Amsterdam, NY) was run with each batch of foods regardless of detection method. If the determined concentrations of the control sample varied by more than 2.0 standard deviations, the entire sample batch was rejected and rerun. Data were reported as mean ± standard deviation.

RESULTS AND DISCUSSION

Processed Pork Products - The vitamin K contents in processed pork products are shown in **Table 1**. Only a small amount of phylloquinone was detected in Kielbasa and pork sausage, which was expected as phylloquinone is plant-derived, hence primarily found in plant-based foods, including certain oils. In contrast, the menaquinone-10 content ranged from 289 to 492 μg/100 g in Kielbasa and pork sausages. In contrast, the menaquinones content of Canadian bacon was <5 μg/100 g. The fat contents of processed pork products were strongly associated with total vitamin K (r=0.9350) (**Figure 3**). The pork sausages contained high amounts of fat (18-32 g/100 g) (**Table 1**), compared to Canadian bacon, which only contained ~3 g fat/100 g. No effect on the menaquinone contents was noted in response to either pan-frying or grilling, which is consistent with a previous study that reported phylloquinone and menaquinone-4 to be cooking-stable.[17]

Fresh Pork Cuts - Phylloquinone and 10 menaquinone forms were analyzed in six fresh-cuts of pork (**Table 2**). Phylloquinone was not detected in any of the fresh pork cuts. For further verification of non-detectable amounts of phylloquinone, we analyzed each sample using both the APCI-LC-MS and HPLC with fluorescence detector, the latter having greater assay sensitivity for phylloquinone. In contrast, every sample contained menaquinone-4. Menadione is the primary dietary source of vitamin K in domestic swine feed.[18] Menadione is absorbed by intestine and transported to tissues where menadione is converted into menaquinone-4 by the prenylating enzyme UBIAD1.[19] Therefore it is most likely that the menaquinone-4 in fresh pork cuts originates from the menadione in swine feed.

More surprising was the observation that fresh pork cuts contained large amounts of individual forms of menaquinones, especially menaquinone-10 and menaquinone-11. There was also wide variation in menaquinone-10 among various pork cuts, with spareribs containing ~43 µg/100 g compared to <1.0 µg/100 g in tenderloin. Menaquinone-11 had similar variation among the cuts. Menaquinone-9 was also detected in the various fresh pork cuts, albeit at lower concentrations compared to menaquinone-10 and menaquinone-11. Even though vitamin K values are much lower than those for processed pork products, all of these could provide a significant percentage of the dietary recommended intake for vitamin K should these forms be shown to have equivalent bioavailability to phylloquinone. Unfortunately we did not have corresponding fat contents of the fresh pork cut samples to determine the contribution of fat content to variability in menaquinone contents in these samples.

There is no established recommended daily allowance for vitamin K due to the insufficient data available to generate this precise recommendation. Instead, an adequate intake (AI) has been determined, which is 90 and 120 µg/day for women and men, respectively.[20] The AI is based on our knowledge of phylloquinone intake in healthy populations in part because various population estimates of phylloquinone intake is e more accurate than intake of menaquinones due to an overall lack of food composition data of the latter.[21-23] An average serving size of sausage is about 85 g, which contains 250-450 µg of menaquinones. If these menaquinone forms have equivalent bioavailability to phylloquinone, then fresh pork and processed pork products may be important contributors to overall vitamin K intake. Unfortunately, few reports have directly compared the bioavailability of phylloquinone and menaquinone forms.[24-26] Menaquinone 4-11 are detected in rat plasma and livers about 6 hours following a single oral dose of each form, respectively.[24] However, there was variable response of menaquinones to support blood coagulation, with menaquinone-9 only showing an effect on blood coagulation 24 h after oral administration. When the absorption of phylloquinone, menaquinone-4 and menaquinone-9 were compared in equimolar amounts in humans, serum concentrations of phylloquinone was much higher than those of menaquinone-4 and menaquinone-9.[25] Although there results suggest that phylloquinone has better intestinal absorption than menaquinone-4 and menaquinone-9, menaquinone-9 had a much longer half-life than phylloquinone. To the best of our knowledge, there are no studies that have examined bioavailability of menaquinone-10 and 11 compared to phylloquinone in human. In the absence of robust bioavailability data, one cannot conclude the relative contribution of the menaquinone forms found in pork to total vitamin K intakes in the U.S. population.

Interestingly, these same menaquinone forms in pork have been identified in the livers of cows and humans, but the origin of these menaquinone forms has been a source of speculation.[27] Beef liver and kidney are known to contain menaquinone-10 and menaquinone-11.[27] Certain fermented milk products and blue cheese also contain menaquinone-10.[28] The prevailing theory is that these menaquinone forms are absorbed from the large intestine where menaquinone forms are produced by gut bacteria.[4] We now posit that menaquinone-10 and menaquinone-11 are present in animal meats, including pork and, may be absorbed following dietary intake and stored in human liver. However, data on metabolism and function of these menaquinone forms obtained from the diet are lacking and we need bioavailability data before making conclusions regarding the dietary contributions of pork to vitamin K status, hence human health.

A major strength of this study is that all processed pork samples were acquired though NFNAP which incorporates a nationally-representative sampling approach. A limitation of this study is that popular pork products in the U.S. food supply, such as ham and hot dogs, have not yet been analyzed for vitamin K.

Conclusion:

This is the first report on phylloquinone and menaquinones contents of fresh-cut and processed pork products and to the best of our knowledge, the first report of large amount of menaquinones found in animal products. The nutritional effect of menaquinones on human health has so far received little attention. However, these data will provide the basis to stimulate research on the role of pork products in providing vitamin K in the U.S. diet, as well as their associations with health outcomes.

Abbreviations used: United Stated Department of Agriculture: USDA; Nutrient Data Laboratory: NDL; National Food and Nutrition Analysis Program: NFNAP; coronary heart disease: CHD; limit of detection: LOD; adequate intakes: AI

Footnote: Supported by the USDA, Agricultural Research Service under Cooperative Agreement No. 58-1950-0-014 and No. 58-1235-1-193, and National Pork Board (NPB#14-100).

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Figure captions:

Figure 1. Fresh-cut pork products.

Figure 2. Flow chart diagram of sample collection, preparation and analysis.

Figure 3. The association between vitamin K concentrations and fat contents in the processed pork products.

Tables:

Table 1. Vitamin K contents of processed pork products (µg/100 g).

Food	n	PK	MK4	MK5-9	MK10	MK11	MK12-13	Fat (g/100g)
Kielbasa, unprepared	3	0.8±0.4	21.3±3.8	ND	369±88.7	17.7±6.3	ND	26.4±3.8
Kielbasa, grilled	2	0.8±0.4	23.2±0.3	ND	492±91.2	19.2±2.4	ND	31.7±4.8
Kielbasa, pan-fried	3	0.9±0.4	23.7±5.8	ND	431±89.6	20.4±4.4	ND	28.3±2.5
Pork Sausage, regular fat, unprepared	6	0.8±0.1	27.5±4.1	ND	336±55.9	15.3±2.6	ND	23.5±2.0
Pork Sausage, regular fat, pan-fried	6	0.9±0.3	27.4±6.0	ND	341±80.4	14.9±2.9	ND	27.9±4.4
Pork Sausage, reduced fat, pan-fried	2	2.1±0.5	22.8±6.9	ND	289±20.5	13.3±0.8	ND	18.8±0
Canadian Bacon, unprepared	1	ND	3.4	ND	6.7	40.1	ND	3.16
Canadian Bacon, cooked*	4	0.3±0.1	3.0±1.4	ND	2.0±2.5	29.7±8.6	ND	3

*Fat content was estimated by manufacturer. Phylloquinone: PK; Menaquinone:MK

Table 2. Vitamin K contents of fresh-cut pork (μg/100 g).

Fresh-cut pork	n	PK	MK4	MK5-8	MK9	MK10	MK11	MK12-13
Tenderloin	5	ND	3.7±1.3	ND	0.4±0.2	0.6±0.3	67.5±6.2	ND
Pork chops, boneless	5	ND	5.3±2.8	ND	2.6±1.2	9.2±7.0	51.7±14.6	ND
Pork chop with bone	5	ND	10.1±5.5	ND	4.1±2.3	26.6±15.0	34.0±5.0	ND
Pork back ribs with bone	5	ND	9.7±4.4	ND	3.6±1.4	12.0±4.7	79.5±12.4	ND
St. Louis-style spareribs with bone	5	ND	12.8±7.1	ND	13.9±9.1	43.6±28.0	94.5±28.7	ND
Shoulder blade Boston with bone	5	ND	5.0±4.0	ND	4.8±3.8	8.8±7.0	109±14.4	ND

Phylloquinone: PK; Menaquinone:MK

Figure 1

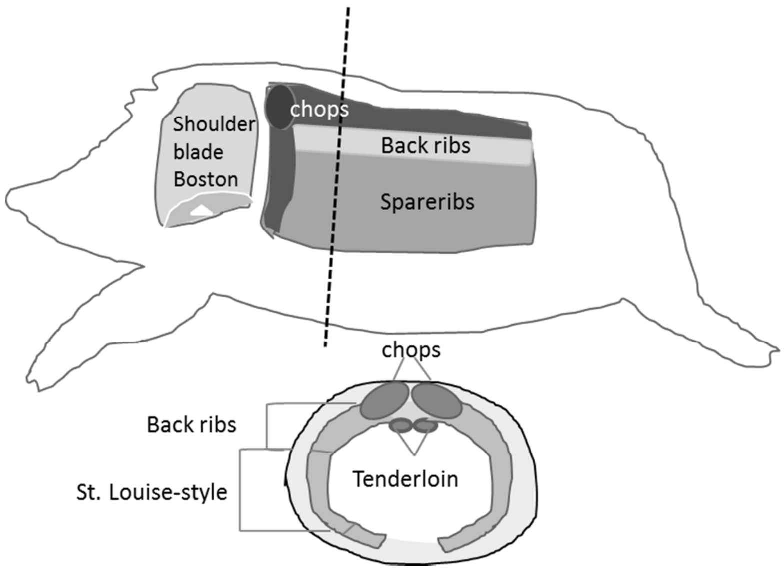

chops
Shoulder
blade
Boston
Back ribs
Spareribs
chops
Back ribs
St. Louise-style
Tenderloin

Figure 2

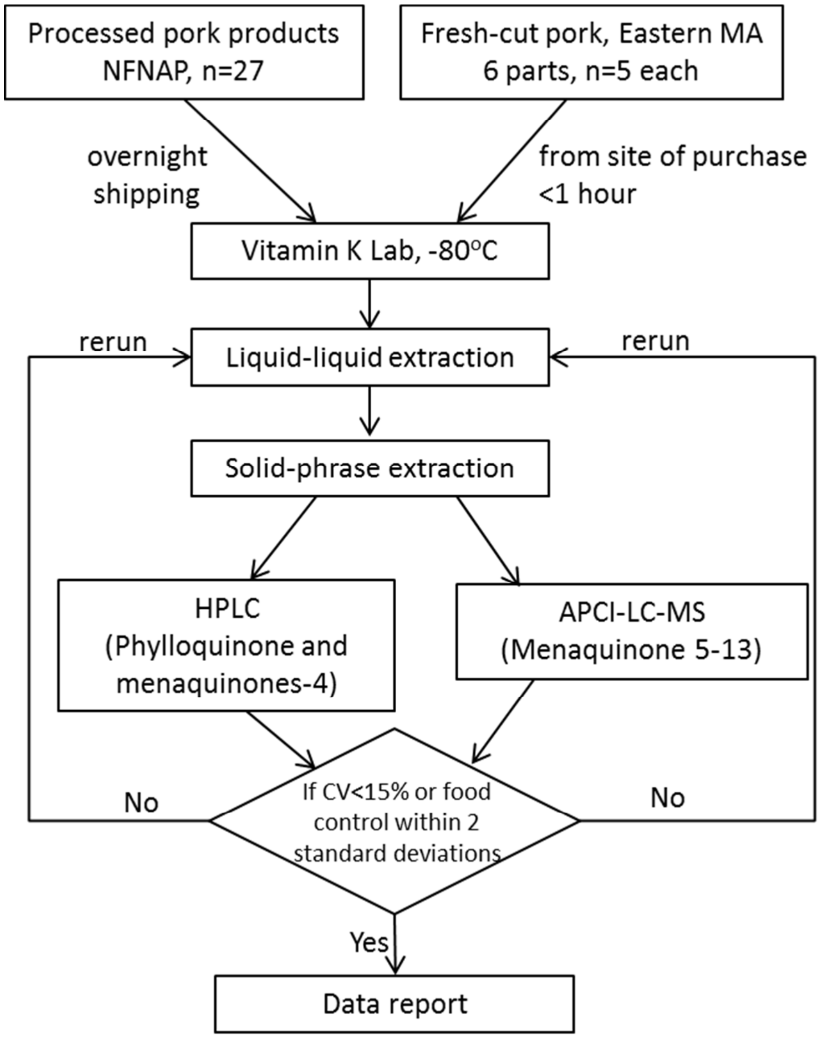
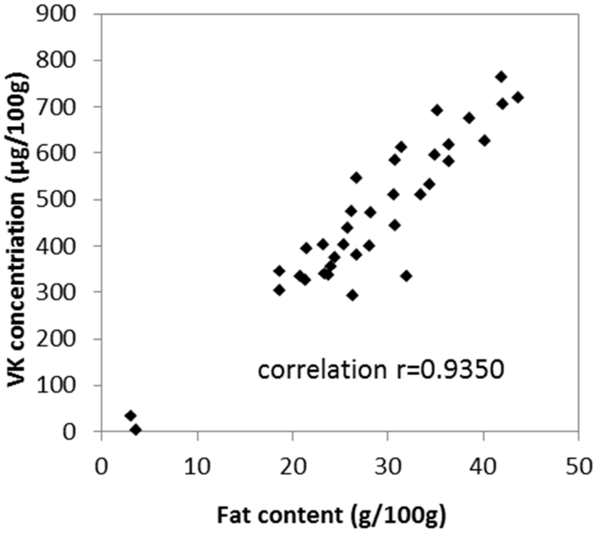

Processed pork products
NFNAP, n=27
Fresh-cut pork, Eastern MA
6 parts, n=5 each
overnight
shipping
from site of purchase
<1 hour
Vitamin K Lab, -80°C
rerun
Liquid-liquid extraction
rerun
Solid-phrase extraction
HPLC
(Phylloquinone and
menaquinones-4)
APCI-LC-MS
(Menaquinone 5-13)
No
If CV<15% or food
control within 2
standard deviations
No
Yes
Data report

Figure 3


900
800
700
600
500
400
300
200
100
0
VK concentriation (µg/100g)
correlation r=0.9350
0
10
20
30
40
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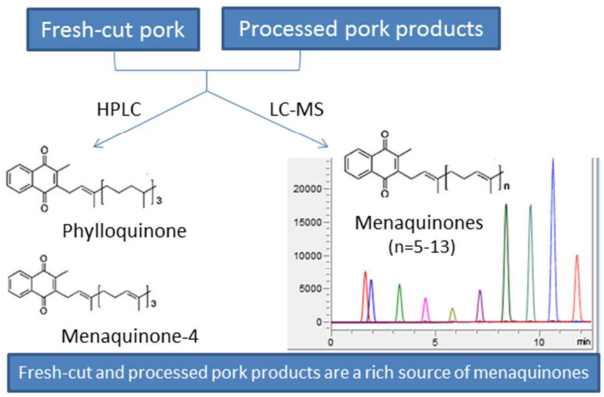
Fat content (g/100g)

TOC Graphic


Fresh-cut pork
Processed pork products
HPLC
LC-MS
Phylloquinone
Menaquinone-4
Menaquinones
(n=5-13)
Fresh-cut and processed pork products are a rich source of menaquinones